LABELING OF GLUCAGON BINDING COMPONENTS IN HEPATOCYTE PLASMA MEMBRANES

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SUMMARY

A photosensitive derivative of glucagon, ^{125}I-N$^{\epsilon}$-4-azido-2-nitrophenyl-glucagon, has been synthesized and used to specifically label glucagon binding proteins in hepatocyte plasma membranes. Photolysis of the derivative in the presence of a membrane suspension results in the incorporation of radioactivity primarily into membrane components with a molecular weight range of 23,000-25,000. The binding properties of the derivative are essentially identical to that observed for glucagon. The binding of ^{125}I-NAP-glucagon was completely inhibited in the presence of glucagon (3 μM) while greater than 90% of the covalent labeling was also inhibited in the presence of glucagon. These studies suggest that the labeled membrane protein may be a component of the glucagon receptor.

INTRODUCTION

The initial site of action of polypeptide hormones has been shown to reside at specific receptor sites on the plasma membrane of various target cells (1-3). The interaction of glucagon with adipocytes and hepatocytes has been shown to result in the activation of adenylate cyclase and the formation of the second messenger, adenosine 3':5'-cyclic monophosphate (4). These studies have been concerned with hormone binding properties, kinetics of interaction, the relationship between binding and the activation of adenylate cyclase, and the isolation and characterization of receptor proteins (1-6).

Abbreviation used: NAP-glucagon, N$^{\epsilon}$-4-azido-2-nitrophenyl-glucagon.

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In an effort to define and covalently label the glucagon receptor components *in situ*, use has been made of the technique of photoaffinity labeling. This technique has been used to specifically label functional sites in several membrane systems (7-15). In this communication we report the use of a photosensitive ε-amino-arylazide derivative of glucagon (NAP-glucagon) to specifically label the hormone binding components in hepatocyte plasma membranes.

MATERIALS AND METHODS

Crystalline glucagon was obtained from Sigma Chemical Co.; [8-^{14}C]-3', 5'-cyclic adenosine monophosphate was obtained from New England Nuclear; [8-^{3}H]adenosine triphosphate and Na^{125}I were purchased from ICN Chemical and Radioisotope Div.; acrylamide and methylenebisacrylamide were purchased from Bio-Rad Laboratories. All other chemicals were of reagent grade. Glucagon (10 mg) was dissolved in 50 mM sodium borate (12 ml) and the pH adjusted to 10.2. This solution was treated with 4-fluoro-3-nitrophenylazide (20 mg) dissolved in ethanol (6 ml), and the reaction stirred for 24 h at 60° C, protected from light. The product was precipitated with ether (40 ml), desalted on Sephadex G-10, dissolved in 7 M urea-0.01 M Tris (pH 7.7), and purified on a DEAE-Sephadex A-25 column (1.2 X 15 cm) at 5° C. Unreacted glucagon was eluted from the column in the absence of NaCl, while NAP-glucagon was eluted with 0.15 M NaCl and finally purified on a Sephadex G-10 column. This product was shown to be modified by the arylazide moiety on the ε-amino group of lysine-12. The characterization and physical properties of NAP-glucagon will be presented elsewhere (16).

Purified hepatocyte plasma membranes were prepared from the livers of male Sprague-Dawley rats fed *ad libitum*, as previously described, using a polymer two-phase system (17). The purity of the membrane preparation was ascertained by analysis of activities of marker enzymes for plasma membranes (5'-nucleotidase [17]), endoplasmic reticulum (glucose-6-phosphatase [18]) and mitochondria (succinate dehydrogenase [19]). Plasma membranes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (20). Iodination of glucagon and NAP-glucagon was carried out using Na^{125}I and chloramine-T as previously reported (21). Gels were stained for protein with Coomassie brilliant blue according to Fairbanks *et al.* (22). Molecular weight estimations were obtained from a parallel calibration gel utilizing ribonuclease, β-lactoglobulin, chymotrypsinogen A, aldolase, ovalbumin, bovine serum albumin, transferrin, phosphorylase a and thyroglobulin as standard marker proteins. Protein was determined by the method of Lowry *et al.* (23) using bovine serum albumin as a standard. The distribution of ^{125}I was determined by slicing the gels into 1 mm discs using a Misco 3015 slicer and counting the hydrogen peroxide-treated gel slices in a scintillation cocktail containing 25% Triton X-100 and 0.4% Butyl-PBD fluoralloy in toluene on a Beckman LS-245 liquid scintillation counter.

Photolysis of ^{125}I-NAP-glucagon (1.5 nM; 2 μCi) in the presence of hepatocyte membranes was carried out in a glass water-jacketed vessel at 30° C for 10 min using a General Electric medium pressure 400 watt mercury arc lamp, as previously described (13). Following photolysis, the membranes were washed and prepared for gel electrophoresis. The binding of ^{125}I-glucagon and ^{125}I-NAP-glucagon to hepatocyte plasma membranes was measured as preiously described (24) using a Brinkmann 3200 microcentrifuge. Adenylate cyclase activity was measured essentially according to the procedure of Storm *et al.* (25).

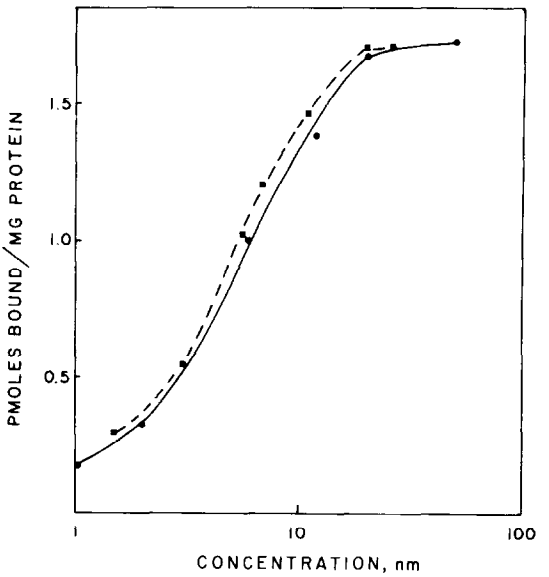

Fig. 1. Binding of ^{125}I-glucagon (■- -■) and ^{125}I-NAP-glucagon (●—●) to hepatocyte plasma membranes. Samples containing 600 μg of membrane protein/ml were incubated in the dark for 10 min at 30° C in Tris·HCl (pH 7.5) containing 1% BSA and 18 mM Mg^{2+}. Aliquots (25 μl) were removed and membrane-bound ^{125}I-glucagon or ^{125}I-NAP-glucagon analyzed by centrifugation. Specific binding was determined after incubating in the presence of glucagon (3-10 μM) prior to the addition of the iodinated hormones.

RESULTS AND DISCUSSION

The binding characteristics of ^{125}I-NAP-glucagon in the absence of light was determined in order to assess the significance of the photolabeling results. The specific binding of glucagon and the glucagon derivative to hepatocyte plasma membranes as a function of hormone concentration is shown in Fig. 1. These results suggest that the photosensitive derivative binds in an identical fashion as the native hormone. Furthermore, the binding of ^{125}I-NAP-glucagon (1-10 nM) was completely inhibited when 3 μM glucagon was added prior to the addition of the photoprobe, again supporting the fact that the photoderivative was binding to the same site(s) as the native hormone.

The addition of glucagon (3 μM) to the hepatocyte plasma membrane preparation resulted in a 3.3-fold stimulation of adenylate cyclase activity. NAP-glucagon, however, did not stimulate adenylate cyclase activity, although its

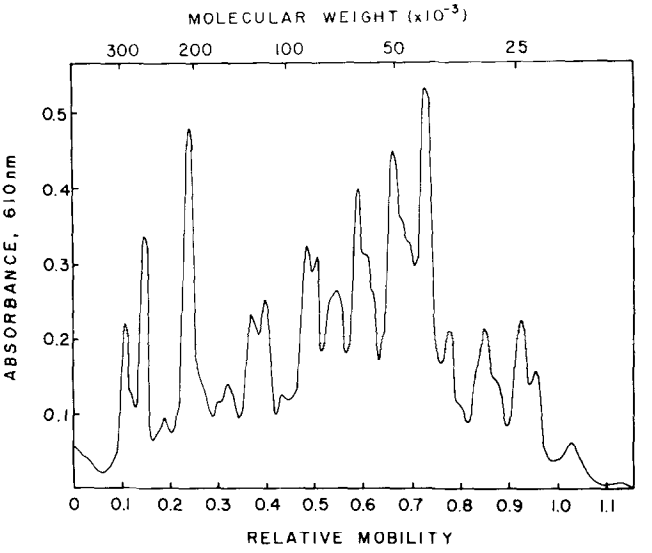

Fig. 2. Densitometer scan of 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis of hepatocyte plasma membranes. Coomassie blue stained gel contained 120 μg of membrane protein.

binding properties were identical to glucagon. Several glucagon derivatives (26,27) have also been shown to exhibit a greatly impaired ability to stimulate this enzyme. Des-histidine-glucagon, as synthesized by the one-step Edman degradation described by Rodbell (28), has also been shown not to activate the adenylate cyclase system in liver. It has been suggested that this derivative is also modified at the ε-amino group of lysine by the phenylthiocarbamyl moiety (26). Despite the absence of biological activity, this derivative has been shown to be a competitive inhibitor of glucagon action (29) and glucagon binding (28), and to induce coupling between the receptor and adenylate cyclase (30,31).

Hepatocyte plasma membranes were purified and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A densitometer scan of a gel stained with Coomassie blue is shown in Fig. 2. Enzymatic marker analysis showed the membrane preparation to be relatively free of contamination by endoplasmic reticulum and mitochondria and to exhibit a 30-fold purification

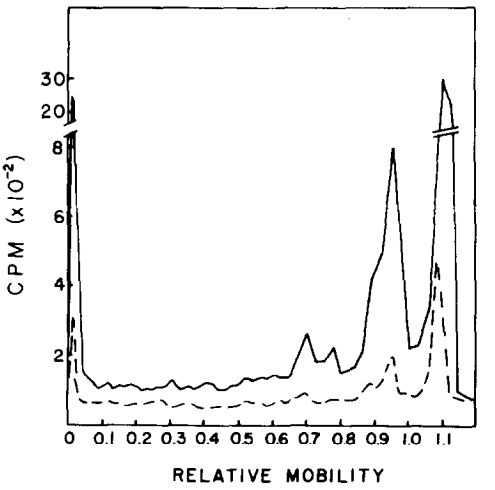

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of hepatocyte plasma membranes which have been photolyzed in the presence of ^{125}I-NAP-glucagon (———). Membranes (600 μg) were suspended in 1 ml of 20 mM Tris·HCl (pH 7.5), 1% bovine serum albumin and 18 mM $MgCl_2$. After a 10 min incubation in the dark, at 30° C, with ^{125}I-NAP-glucagon (1.5 nm; 2 μCi), the suspension was irradiated for 10 min at 30° C. 100 μg of washed membrane was electrophoresed on 7.5% gels. A parallel photolysis was performed where glucagon (3 μM) was added prior to the addition of the photosensitive derivative (– – –).

as measured by 5'-nucleotidase. Plasma membranes were irradiated in the presence of ^{125}I-NAP-glucagon for 10 min, at 30° C, after a 10 min pre-incubation in the dark. The membranes were washed and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, these results shown in Fig. 3. Two membrane components were significantly labeled with a molecular weight range of 23,000 - 25,000. A high molecular weight component was also labeled, which was shown to be primarily an aggregate of a protein with a molecular weight of 25,000. The details of this observation will be presented in a subsequent report. Radioactivity running ahead of the tracking dye (bromphenol blue) represents glucagon which was not covalently attached to the membrane. When the irradiation was carried out in the presence of glucagon (3 μM), a decrease in the incorporation of radioactivity of greater than 90% was observed in all of the labeled components, supporting the binding data

and suggesting a high degree of specificity in the photolabeling reaction. The appropriate controls also showed that a) photolysis was required for covalent incorporation of the probe; b) irradiation of pre-irradiated ^{125}I-NAP-glucagon in the presence of hepatocyte membranes resulted in no incorporation of radioactivity; and c) the incubation and photolytic conditions had no effect on the Coomassie blue gel patterns.

Several studies have shown that membrane associated receptors for acetylcholine (32,33), thyrotropin (34) and insulin (35) are composed of multiple components. The binding of ^{125}I-glucagon to a Lubrol-PX extract of cat myocardial tissue has been reported (36). Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated the presence of a glucagon binding protein with a molecular weight of 24,000 - 28,000. Lubrol-PX extraction of rat hepatocyte plasma membranes afforded a glucagon binding component with a molecular weight of 190,000, however, under non-dissociating conditions (37). These results also suggest the possibility that the glucagon receptor may be composed of multiple subunits. The results of this report suggest that the covalently modified membrane proteins (23,000 - 25,000 daltons) from hepatocyte plasma membranes may be components of the glucagon receptor.

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